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RESEARCH ARTICLE

Neutralizing activity to SARS-CoV-2 in 1.2 to 10.0 month convalescent plasma samples of COVID-19: A transversal surrogate in vitro study performed in Quito-Ecuador

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Abstract

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, was first reported in Wuhan, China, in December 2019. Diagnostic methods for the detection of the virus and seroconversion of neutralizing antibodies (NAbs) in plasma have been developed specifically, but some of them require a BSL3 facility. In this study, we used the SARS-CoV-2 Surrogate Virus Neutralization Test Kit to determine the presence or absence of NAbs anti-receptor binding domain of the viral spike (S) glycoprotein in a BSL2 facility. The sample population was chosen in Quito, Ecuador, with a total of 88 COVID-19 positive convalescent patients. We determined that 97.7% of the analyzed convalescent sera maintained the presence of NAbs with neutralizing activity, and this activity remained until 10 months after the infection in some cases. In addition, the relationship between the presence of NAbs and immunoglobulin G was significant compared to immunoglobulin M, which tended to be absent over time.

KEYWORDS
convalescence, IgG, IgM, neutralizing antibodies, plasma, SARS-CoV-2

1 | INTRODUCTION

In December 2019, the first cases of pneumonia caused by a novel coronavirus were reported in Wuhan, China.[1] On the 11th of February, the World Health Organization (WHO) named coronavirus disease 2019 (COVID-19), was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[2] It was considered a pandemic on March 11, 2021.[3] By April 2022, COVID-19 had affected at least 200 countries. To date, more than 500 million cases and more than 6.2 million deaths have been confirmed worldwide.[4]

Coronavirus belongs to the family Coronaviridae suborder of Coronavirinae within the order Nidovirales, and belongs specifically to the Betacoronavirus group. It is an enveloped virus with positive-sense, single-stranded RNA.[5] It is well known that SARS-CoV-2 strains are highly contagious, spread rapidly, and continuously mutate around the human population; thus, they have become an international public health problem.[6,7]

Viral infection occurs when human receptors such as aminopeptidase N (APN; HCoV-229E), angiotensin-converting enzyme 2 (ACE2; HCoV-NL63), and dipeptidyl peptidase 4 (DPP4) are specifically recognized by the viral spike (S) surface glycoprotein before entering the host cell.[5] After endocytosis, SARS-CoV-2 liberates its genomic RNA, and the cell expresses viral genes to produce countless viral structures that self-assemble to produce viral particles.[8] Receptor interactions trigger irreversible conformational changes in cell peak proteins that allow membrane fusion during viral infections.[9,10] ACE2 receptors are found in enterocytes,[11–13] ciliated

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alveolar epithelium, nasopharynx cells, stomach, liver, and kidney in human cells.[14,15]

Neutralizing antibodies (NAbs) against SARS-CoV-2 are generated specifically by B lymphocytes after the entrance of an antigen, such as a pathogen, or by vaccine technologies.[8,11,16] Among their features and benefits, they can be used as a tool for analyzing humoral immunity, preventing and treating diseases such as immunodeficiencies, hematological and inflammatory diseases, neuromuscular disorders, certain infections, autoimmune diseases, and allergies.[17–23] Monoclonal NAbs against SARS-COV-2 can target the spike (S) viral protein, thus neutralizing the antigen completely and hence viral entry into the host cell.[9]

Several publications have detailed valuable information about detection, immunization, and viable effective treatments using immunoglobulins (Igs), also called antibodies.[9,17,18,21,24,25] Igs are produced in the humoral adaptive system and have remarkable biological effects against pathogens by neutralizing the infection and assisting phagocytic cells, since they can opsonize antigens, mediate antibody-dependent cellular cytotoxicity, activate the complement system that lyses pathogens in infected cells, and agglutinate antigens. Immunoglobulin M (IgM) is the primary antibody produced to address a new infection. Immunoglobulin G (IgG) is expressed only a few days after infection or vaccination.[22,26]

In vivo neutralizing assays, which are the gold standard for studying viral infections, require special Type-III laboratory facilities. Some serological tests, such as lateral flow devices (LFDs) and blocking enzyme-linked immunosorbent assay (ELISA) assays for detecting antibodies or their neutralization activity, respectively, have proven to be as sensitive as reverse transcription-polymerase chain reaction (RT-PCR) or in vivo infection assays.[23,25,27–29] Subsequently, these immunological assays can be performed as screening methods for type II laboratories. Reports of humoral memory response show that IgM and IgG levels decrease over a period of 1.3 and 6.2 months, however, they are still active.[11] In addition, at 6 months postinfection, antibody-neutralizing activity was reported to have remained stable.[30] NAbs that recognize the viral spike (S) protein of SARS-CoV-2 can persist for at least 5 months postinfection.[31] Similarly, recent studies have detected NAbs with a half-life of 31.4 weeks,[32] 10 months,[33] and up to 12 months[34] where they were measured in convalescent individuals. These studies suggest that reinfection may be less likely than currently feared.[23,35] However, as the pandemic continues, more studies should be conducted to understand the prevalence of antibodies and humoral immunity.

The purpose of this study, realized in Quito-Ecuador, is to transversally analyze, in vitro, the neutralizing activity and persistence of both IgG and IgM antibodies to SARS-CoV-2 in positive COVID-19 convalescent plasma samples (CPS) analyzed at different times, ranging from 1.2 to 10.0 months, in a BSL2 facility. These findings and correlations will contribute to the understanding of humoral metabolism and the neutralization potential of CPS for therapeutic antibody treatments or vaccination technologies.

2 | METHODS

2.1 | Study design, ethical considerations, and sample collection

This was an observational, cross-sectional study in a small cohort that included adults (>18 years old) from Quito-Ecuador. This study was approved with the protocol number 097-2021, document number MSP-CGDES-2021-0207-O by the Ethics Committee for Expedited Review of COVID-19 Investigations of the Ministry of Public Health (MSP) of Ecuador in accordance with the Declaration of Helsinki. Data from patients diagnosed as positive for SARS-CoV-2 were obtained from the Centro de Atención Quito Solidario (CAT) from July to August 2020, and from the Hospital de Servicios Odontológicos (HSO) from July to December 2020.

Sample collection was performed at the HSO facilities. Selected individuals from the databases were contacted, arrived at the HSO facilities, and signed the informed consent previously approved, which allowed the collection of data and blood samples. Blood samples were aseptically extracted using a blood needle holder via venous blood vacuum extraction and collected in heparinized blood collection tubes (Vacutainer). After the collection, the samples were centrifuged at 1000 *g* for 10 min and kept at 4°C for transportation. Every sample was anonymized by creating a code that contained the patients' name initials and the first four digits of their citizenship identification number. The samples were transported to the Molecular Biology and Genetics laboratories at the Hemisferios University, where 1 ml of separated serum was aliquoted into 1.5 ml microtubes and finally stored at −20°C for their later use.

2.2 | ELISA SARS-CoV-2 surrogate neutralization assay

A blocking ELISA reaction was performed to detect the presence or absence of NAbs anti-RBD of the viral spike (S) glycoprotein. Plasma samples were analyzed using the SARS-CoV-2 Surrogate Virus Neutralization Test Kit (GenScript). Samples were processed according to the manufacturer's recommendations. Briefly, the horseradish peroxidase-receptor binding domain (HRP-RBD) solution was prepared by adding 10 µl of HRP-RBD conjugated in 9.99 ml of HRP dilution buffer. At the same time, diluted samples and control samples (positive and negative) were prepared by separately mixing 10 µl of each sample in 90 µl of sample dilution buffer. Immediately, 60 µl of the previously prepared patient and control plasma-samples were mixed with the HRP-RBD solution and incubated at 37°C for 30 min. Next, 100 µl of each plasma-sample and both positive and negative controls were added to the corresponding wells and incubated for 15 min at 37°C. After this, the plate was washed four times with 260 µl of 1X wash solution. TMB Solution (100 µl) was added to each well and the plate was incubated in the dark at room temperature (20–25°C) for 15 min. Finally, the reaction was quenched by adding

50 µl of stop solution, and the absorbance of each well was read in a Sirio-S RADIM diagnostics microtiter plate at 450 nm.

Anti-SARS-CoV-2 neutralizing activity for all convalescent plasma-samples was reported qualitatively as positive or negative inhibition by following the SARS-CoV-2 Surrogate Virus Neutralization Test Kit specifications, where positive and negative inhibitions showed values ≥20% and <20%, respectively. Quality control for both positive and negative controls, provided in the surrogate neutralization assay kit, was corroborated by measuring their optical density.

2.3 | Immunochromatographic lateral flow assay for detecting IgG and IgM anti-SARS-CoV-2 S1 subunit antigen

The presence or absence of IgG and IgM anti-SARS-CoV-2 S1 spike (S) viral protein was assessed using the Spring Healthcare ERC-SG5257 COVID-19 Rapid IgG/IgM Combined Antibody Assay Pre-Screening Kit (Shanghai ZJ Bio-Tech) according to the manufacturer's recommendations. One drop of each plasma sample (positive and negative controls) was transferred to the specimen well of the LFD with a disposable pipette provided by the kit manufacturer. Then, one drop of the buffer solution was added to the same well, and after 10–15 min, the results were read following the manufacturer's specifications.

2.4 | Data reporting and statistical analyses

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 26.0. Data normality was evaluated using the Kolmogorov–Smirnov test. Nonparametric continuous variables were analyzed as medians and interquartile ranges, and parametric variables were presented as means ± standard deviations. The Mann–Whitney *U* test was applied to compare the medians between groups of nonparametric variables. In addition, the phi correlation coefficient was employed to compare dichotomous variables. An analysis of variance test, followed by the Student's *t* test, was performed to compare the means of parametric variables. Categorical variable frequencies were compared using Pearson's χ^2 test.

3 | RESULTS

3.1 | Study cohort characteristics

Eligible participants, diagnosed as COVID-19 positive, were selected from the databases of CAT and the HSO, where the disease was confirmed either by SARS-CoV-2 RT-PCR assay, immunochromatography lateral flow assay for IgG and IgM anti-SARS-CoV-2, and/or by a combination of novel coronavirus disease 2019 related symptom onset. Conversely, samples from three participants from CAT databases, diagnosed as COVID-19 negative by SARS-CoV-2 RT-PCR and COVID-19 rapid IgG/IgM antibody methods combined with COVID-19 symptomatology, were selected for this study as COVID-19 negative convalescent samples (NPS). A total of $N = 91$ plasma samples from eligible participants were collected at the beginning of July 2021, and 88 were categorized as CPS. The convalescence period of the samples ranged from 1.2 to 10.0 months from the manifestation of COVID-19 symptoms. Cohort age ranged from 19 to 87 years, and the sex distribution was 48 men and 43 women (Table 1).

TABLE 1 Participant demographics

Sample size	Count/%
COVID-19 convalescent plasma samples (CPS)	88.0 (96.7%)
Diagnosed by PCR, related symptoms and/or lateral flow devices (LFD)	62.0 (68.1%)
Diagnosed by LFD and related symptoms	19.0 (20.9%)
Diagnosed by related symptoms only	7.0 (7.7%)
COVID-19 non-convalescent plasma samples (NPS)	3.0 (3.3%)
Convalescence time	Months
Mean	5.7
Mode	6.8
Min	1.2
Q1	5.2
Q2 (Median)	6.0
Q3	6.8
Max	10.0
Antibody detection in COVID-19 CPS	Count /%
Neutralizing antibodies (NAbs)	86.0 (97.7%)
Immunoglobulin G (IgG)	86.0 (97.7%)
Immunoglobulin M (IgM)	23 (26.1%)
Age	Years
Mean	48.3
Mode	53.0
Min	19.0
Q1	37.0
Q2 (Median)	49.0
Q3	58.5
Max	87.0
Sex	Count /%
Male	48.0 (52.7%)
Female	43.0 (47.3%)

3.2 | Antibodies distribution in the study population, $N = 91$ plasma-samples

Neutralization activity against the union of the viral-RBD of the spike (S) protein to the ACE2 host cell receptor was found positive in 94.5% (86 of 91 plasma samples) of the study population. Similarly, 94.5% of plasma samples reacted positively to the qualitative immunochromatographic lateral flow assay when detecting IgG anti-SARS-CoV-2 spike (S1) antigen. In addition, IgM presence was detected qualitatively in 25.3% (23 of 91 plasma samples) of the participants' blood samples (Table 1). Conversely, neither neutralizing activity nor IgG and IgM presence were found in all NPS (3 of 91 plasma samples), as expected. The χ^2 data analysis described in Table 2 shows that the seroconversion of IgG anti-SARS-CoV-2 spike (S1) antigen and NAbs anti-RBD spike (S) viral purified protein differed significantly ($p < 0.001$) between CPS and NPS.

TABLE 2 Production of immunoglobulin G anti-SARS-CoV-2 S1 subunit and detection of neutralizing activity to purified RBD spike (S) viral glycoprotein, in COVID-19 negative plasma samples (NPS) and COVID-19 convalescent plasma samples (CPS). $N = 91$

Antibodies	NPS $n = 3$	CPS $n = 88$	p
IgG[a,b]	0 (0%)	86 (97.7%)	<0.001
NAbs[b]	0 (0%)	86 (97.7%)	<0.001

Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
[a]n = Frequencies, χ^2;
[b]Presence.

3.3 | COVID-19 CPS analysis

3.3.1 | Neutralizing activity to SARS-CoV-2 RDB from the spike (S) purified protein persists in CPS

The surrogate neutralization in vitro assay showed that 97.7% (86 of 88 plasma samples) of CPS ($n = 88$) inhibited the union of the purified proteins RBD-S protein and ACE2 receptor (Figure 1). The CPS convalescence times varied between 1.2 and 10.0 months. In 2 of 88 (2.3%) CPS the neutralizing activity was found to be null or not detectable, and the convalescence times were 2.6 (female, 53 years old) and 6 (male, 50 years old) months, respectively.

3.3.2 | Distribution of IgG and IgM anti-SARS-CoV-2 S1 subunit antigen in 1.2 to 10.0 month CPS

The Spring Healthcare SARS-CoV-2 Antigen Rapid Test Cassette allows the qualitative determination of the presence or absence of IgG and IgM anti-SARS-CoV-2 S1 subunit antigen of the viral spike protein, with high specificity and sensitivity.[27,28] In Figure 1, the distribution of IgG shows that it was expressed in the majority of the samples, representing 97.7% (86 of 88) of CPS. On the other hand, IgM was detectable in 26.1% of CPS (23 of 88). CPS had different convalescence times, ranging from 1.2 to 10.0 months. The absence of IgG found in 2 CPS (males, convalescence times of 6.0 and 9.6 months, aged 50 and 46 respectively), representing 2.3% of the population. IgM was not detectable in 65 (73.9%) of the CPS (Table 1).

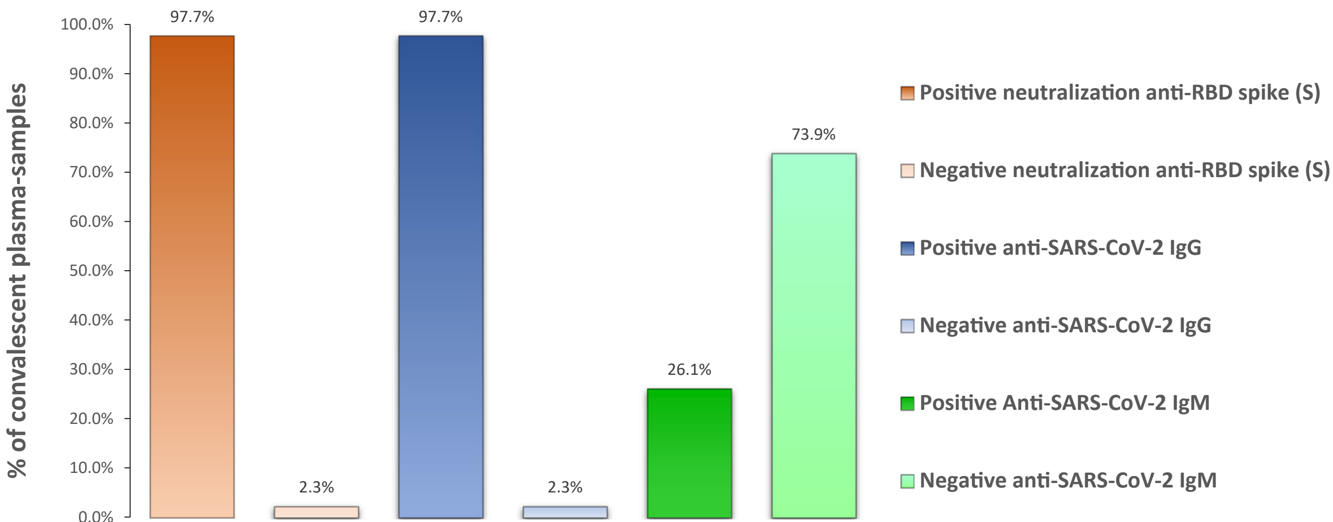

FIGURE 1 Immunological assays performed in $n = 88$ COVID-19 convalescent plasma samples (CPS) ranging from 1.2 to 10.0 months. The distribution of neutralizing antibodies (NAbs) and immunoglobulins G (IgG) and M (IgM) was determined by a qualitative in vitro blocking ELISA assay for the detection of neutralizing activity against the union of the SARS-CoV-2 purified receptor binding domain (RBD) with the purified angiotensin-converting enzyme 2 receptor (ACE2) and an immunochromatic lateral flow assay (LFD) for the detection of IgG and IgM anti-SARS-CoV-2 S1, respectively. COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

TABLE 3 Correlational analysis between the expression of neutralizing antibodies (Nabs), IgG, and IgM in 1.2–10.0 COVID-19 convalescent plasma samples (CPS)

Antibodies	NAbs	IgG	IgM
NAbs	1	$r_\phi = 0.488$[a]	$r_\phi = 0.091$[a]
		$p < 0.001$*	$p = 395$
IgG		1	$r_\phi = 0.091$[a]
			$p = 395$
IgM			1

Abbreviation: COVID-19, coronavirus disease 2019.

*Significant correlation at the 0.01 level.

[a]Correlation coefficient Phi (φ).

3.3.3 | Positive correlation of the neutralizing activity to RBD of SARS-CoV-2 spike (S) protein and the seroconversion of IgG was detectable in CPS

The correlational phi coefficient analysis applied between NAbs, IgG, and IgM variables showed that the CPS' neutralizing activity against the RBD of the SARS-CoV-2 spike (S) protein and the expression of IgG anti-SARS-CoV-2 S1 subunit antigen had a strong positive relationship ($r_\phi = 0.488$) with a significance of $p < 0.001$ (Table 3). On the other hand, no strong relationship was observed when comparing the expression of NAbs and IgM, as well as between both IgG and IgM ($r_\phi = 0.091$ in both cases), as shown in Table 3.

3.3.4 | The expression of IgG/IgM anti-SARS-CoV-2 S1 subunit antigen and NAbs to the RBD of the spike (S) SARS-CoV-2 protein is independent of the age and sex of COVID-19 patients

CPS ($n = 88$) was analyzed to determine the correlation of the age and sex of participants compared with the seroconversion of IgG, IgM, and NAbs, independently. The CPS convalescence times ranged from 1.2 to 10.0 months. As expected, the expression of the antibodies assessed in this study was not dependent on the age or sex of COVID-19 convalescent participants (Tables S1 and S2).

3.3.5 | The seroconversion of IgM anti-SARS-CoV-2 S1 subunit antigen CPS declines over time

The presence of IgM anti-SARS-CoV-2 S1 viral antigen was detected qualitatively. A significative difference ($p < 0.001$) was found when transversally analyzing the presence of IgM during different CPS times that ranged between 1.2 and 10.0 months ($n = 88$). Statistical analysis showed that the seroconversion of IgM was undetectable or null after 4.78 months (median = 6.23; Table S3).

4 | DISCUSSION AND CONCLUSION

The conventional neutralizing assays for assessing immunity involve handling cells and active viral particles in centers with specialized biosafety level 3 (BSL3)[25] restrains the investigation in countries lacking these facilities. The employment of well-validated surrogate blocking-ELISA neutralizing assays and LFD,[28,29,36,37] such as the SARS-CoV-2 Surrogate Virus Neutralization Test Kit (GenScript)[25] and COVID-19 Rapid IgG/IgM Combined Antibody Assay Pre-Screening Kit (Spring Healthcare)[27] respectively, which could be performed in BLS2 facilities, represents an alternative for measuring and evaluating the persistence of NAbs to SARS-Cov-2 in any country with BSL2 facilities.

The surrogate neutralization assay mimics, in vitro, the viral infection by assessing the union of RBD with ACE2 receptors rather than looking specifically for NAbs.[25] It is known that NAbs diminish with time, but specific B cells are still active and produce antibodies with greater somatic resistance to RBS mutations and potency.[11,38] The transversal analysis in this report showed that 97.7% of CPS could inhibit the union of the viral S1-RBD protein to the ACE2 receptor in plasma samples, whose convalescence times ranged between 1.2 and 10.0 months. Other studies found that neutralization to SARS-CoV-2 is still detectable during 2, 4, 5, 6.2, 7.8, 9, and 12 months after symptoms onset when samples were examined.[11,23,30,32–34,36,39,40] IgG targeting the viral S1 subunit or its RDB can block viral entry into the cell host,[16,23,41] and IgM and IgA have also been reported to have the same affect.[42] Similarly, the results of the statistical analysis demonstrated that the presence of IgG anti-SARS-CoV-2 S1 protein subunit strongly correlates ($r_\phi = 0.488$) with the neutralizing capability of the CPS even when the seroconversion of IgM anti-SARS-CoV-S1 was null or indetectable after 4.78 months of symptom onset, as expected.[11,40]

The FDA does not recommend the evaluation of immunity or protection against SARS-CoV-2 by testing antibodies at any time or after vaccination, principally if the tests do not detect the same specific Igs whose seroconversion was induced by vaccination.[43] Here, we did not analyze immunity in vaccinated plasma samples, but in CPS, and the ELISA blocking assay (Surrogate Virus Neutralization Test Kit) did not directly detect the presence of antibodies, but the neutralization activity of CPS to the union of RBD-spike and ACE2 purified proteins.[25] The other assays that we performed used LFD (COVID-19 Rapid IgG/IgM Combined Antibody Assay Pre-Screening Kit), which determined the presence/absence of IgG and IgM anti-SARS-CoV-2 S1.[27,28] The S1 subunit and the RBD of SARS-CoV-2 spike (S) glycoprotein are perfect antigen targets when looking specifically for NAbs and/or neutralizing capability, because they play a crucial role during the viral entry phase, mediating the virus attachment to the ACE2 receptor of host cells.[7,9,11,16,23,42,44]

In this study, the neutralizing activity of the CPS analyzed, as well as the seroconversion of IgG and IgM, was found to be independent of age and sex; similar results demonstrated the same correlation.[16,42] In contrast, it was reported that the probability of needing an intensive treatment unit in COVID-19 infected males is higher than that in

women[45]; however, this could not necessarily be due to the lack of neutralizing activity of the blood.

As it is necessary to understand the lifespan and evolution of humoral immunity against SARS-CoV-2, therefore, more investigations and trials should be performed.[37,46] Validated surrogative neutralizing assays, along with LDF as a supplementary test for detecting NAbs such as IgG, IgM, and IgA,[9,11,39,42] are alternatives for characterizing the neutralizing activity of CPS and could be a useful tool in plasma transfusion or monoclonal antibody therapies, which have been proven to reduce the risk of a fatal outcome.[18,39,47,48]

The results presented in this article not only report the neutralization activity of SARS-CoV-2 in COVID-19 CPS but also contribute to the understanding of the duration of antibody-mediated immunity to SARS-CoV-2, principally in the Ecuadorian population where scientific reports related to the novel coronavirus pandemic disease are limited.

AUTHOR CONTRIBUTIONS

Boris Villacrés fulfilled and obtained the Ethics Committee for Expedited Review of COVID-19 Investigations approvement forms, designed the study, performed the experiments, wrote the manuscript (except the introduction), and supervised the project. Elius Paz realized the statistical analysis, collaborated in the bioethical approvement and during the neutralization assay. Irina Villacrés-Granda reviewed, edited, and did the correspondence with this journal. María José Burbano wrote the introduction. María Jose Armijos contacted the participants. Mauricio Aguirre contacted intercourse with donors, funders, and hospitals' directors.

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DATA AVAILABILITY STATEMENT

NA.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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